NASA-CR-200656

FINAL TECHNICAL REPORT: NAG 2-943
Definition phase funding for Neurolab proposal #106
"Microgravity Effects on Developing Vestibular Afferents"
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Results of Definition Phase feasibility studies and flight simulations:

Studies during the definition phase of the project were designed to address four feasibility issues:

1) Can Zebrafish embryos survive in a closed system such as the ECU for the duration of the flight?
2) How early in development can the embryos reach microgravity, given that the eggs must be fertilized on the ground and given the timing constraints of the shuttle launch?
3) Can the fixed embryos remain at 22-24 degrees C for the remainder of the flight without degradation of their morphology?
4) Can the embryos survive the shuttle launch and continue normal development?

Embryo survival in the Egg Chamber Units:

For all egg chamber tests, 50 eggs with chorions enzymatically removed at 80% epiboly were placed in ECUs containing 10% Hanks with 30ug/ml phenylthiourea added to prevent melanin formation. 2 mls paramecium culture was added as a food source and to prevent bacterial proliferation. The chambers were sealed and placed at 22-24 degrees C, and embryo survival was assessed after 2 weeks, the proposed duration of the flight.

In initial tests of the ECUs the chambers were used in three different configurations: first with the air-permeable diaphragms in place, second with the diaphragm removed and the chamber entirely filled. and third with the diaphragm scrunched down near the bottom of the chamber.

Very low or no survival of embryos after two weeks was seen in the first two configurations, presumably due to the small volume in the first case and to lack of oxygen in the second case.

In 10 tests using the depressed diaphragm configuration, embryo survival rates varied from 20-50%, as compared to 50-75% survival for embryos raised under normal laboratory conditions. Thus in a worst case scenario aboard the shuttle there would be 10 specimens collected at each time point, which should be sufficient to fulfill the science requirements.

Because the diaphragm does not always remain well sealed to the sides of the ECU when it is used in the depressed position, the ECU is currently undergoing modification to decrease the height of the diaphragm eliminating the need for this deformation of the diaphragm.

Developmental timing studies:

In order to ensure that the Zebrafish will be able to reach microgravity before the primary vestibular afferents begin to grow into the hindbrain, various cooling paradigms were tested to slow down the embryos development.

Any cooling of the embryos below 22-24 degrees C prior to 80% epiboly was found to be very detrimental to survival. Therefore in all subsequent cooling tests the embryos were raised at
26-28 degrees C until 80% epiboly (~8 hours post-fertilization) before cooling.

Zebrafish raised at 14-16 degrees C or lower either did not survive, or exhibited gross developmental abnormalities of kidney, heart, notochord and brain.

Zebrafish raised at 18 degrees C showed normal survival and developed normally but slowly. The rate of development of the embryos at this cold temperature is about 0.4 times the normal developmental rate. Therefore if a shuttle launch delay results in the maximum waiting period between loading the embryos on board the shuttle and reaching microgravity, a delay of 48 hours, the fish would still be at a developmental age of ~27 hours. At this age the hair cells of the otolithic maculae are just beginning to develop and primary afferent fibers have not yet begun their growth into the hindbrain.

Fixation studies:

In order to determine whether the fixed embryos on board the shuttle could remain in the same Refrigeration/Incubation Module with the still living specimens, or whether refrigeration of the fixed embryos would be necessary, Zebrafish embryos at 1,3,7 and 10 days of development were fixed in 4% paraformaldehyde and left at 22-24 degrees C for 2 weeks. The fixed specimens were then observed at 20 X to check for degradation of gross morphology, and then primary afferent fibers were labeled. Morphology of all the specimens appeared normal, and DiI transport and afferent arbor patterns were unaffected.

Because it is desirable that the volume of concentrated fixative necessary to be injected into the ECUs be as small as possible, and because it is difficult to make very concentrated solutions of paraformaldehyde, further fixation tests are underway to determine whether some or all of the paraformaldehyde can be replaced with some concentration of glutaraldehyde without adverse consequences.

Shuttle launch simulation studies:

In order to determine whether Zebrafish embryos would be adversely affected by the shuttle launch, launch simulation tests were carried out on the 20G centrifuge at NASA's Ames Research Center. In these tests live Zebrafish embryos were subjected to the gravitational profile and acoustic noise levels which occur during an actual shuttle launch.

Four different groups of embryos were tested. Two age groups were used, 20 hours and 28 hours. These ages were chosen because they represent the earliest and the latest stages at which the embryos would experience the launch, depending on whether there is a launch slip or not. The ages were also chosen because it seemed possible that

any adverse effects on survival or gross morphological development might be more pronounced in the younger embryos, while any effects on vestibular system development might be more pronounced in the older specimens where otolith hair cell proliferation had already begun. In each age group a set of normal embryos and a set of embryos with their chorion removed was tested. This test was performed because the revised protocol suggests loading the embryos after removing their chorions, but it was possible that the chorion might provide some degree of protection from any adverse effects of the launch.

One hundred embryos were used in each test group. The embryos were all observed by eye immediately following the launch simulation test, and all appeared to be alive.

At 72 hours the embryos were observed at 20 X and did not appear to have any developmental abnormalities. Gross morphology was normal, as was semi-circular canal wall fusion and otolith development. The inner ears of 10 fish from each group were injected with the specific hair cell label 4-Di-2-ASP in order to assess the development of the vestibular end organs. In all cases the end-organ development appeared normal, with canal cristae and otolith maculae occurring at the normal locations and with normal size and shape.

At one week embryos were sacrificed and fixed in 4% paraformaldehyde, and individual vestibular end-organs in several embryos from each experimental group were injected with DiI to label the primary vestibular afferents. In all cases afferent projection patterns and axonal arborizations appeared normal.

It thus seems that Zebrafish embryos involved in the proposed flight experiments will not be adversely affected by the shuttle launch.